

## Molybdenum(VI) salts convert the xanthine oxidoreductase apoprotein into the active enzyme in mouse L929 fibroblastic cells\*

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The mouse L929 fibroblastic cell line presents low, but detectable, levels of the mRNA encoding xanthine oxidoreductase under basal conditions, and it responds to type I and type II interferons by inducing the expression of the transcript [Falciani, Ghezzi, Terao, Cazzaniga, and Garattini (1992) *Biochem. J.* **285**, 1001–1008]. This cell line, however, does not show any detectable amount of xanthine oxidoreductase enzymic activity, either before or after treatment with the cytokines. Molybdenum(VI) salts, in the millimolar range, are capable of activating xanthine oxidoreductase in L929 cells both under basal conditions and after treatment with interferon- $\alpha$ . The increase is observed in mouse L929 as well as in clones derived from it, but not in many other human and mouse cell lines. The induction observed in L929 cells is post-translational in nature and it is insensitive to cycloheximide, indicating that the molybdenum ion converts a

pool of inactive xanthine oxidoreductase apoenzyme into its holoenzymic form. When grown in the absence of sodium molybdate, the L929 cell line has undetectable intracellular levels of the molybdenum cofactor, since the cell extracts are unable to complement the nitrate reductase defect of the *nit-1* mutant of *Neurospora crassa*. L929 cells grown in the presence of millimolar concentrations of sodium molybdate, however, become competent to complement the *nit-1* defect. L929 cells accumulate molybdenum ion inside the intracellular compartment as efficiently as TEnd cells, a mouse endothelial cell line that expresses xanthine oxidoreductase activity both under basal conditions and after treatment with interferon- $\gamma$ , suggesting that L929 cells have a defect in one or more of the metabolic steps leading to the synthesis of the molybdenum cofactor.

### INTRODUCTION

The xanthine oxidoreductase (XOR) enzymic system catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid. XOR is a dimeric protein which consists of two identical monomeric subunits of 150 kDa each (Rajagopalan and Handler, 1967; Carpani et al., 1990) and it binds 1 molecule of FAD per each subunit (Rajagopalan and Handler, 1967). It is one of the mammalian flavoproteins that require a molybdopteridin cofactor ('molybdenum cofactor') for their activity (Bray, 1975, 1982, 1988; Johnson, 1980). The structure of the molybdenum cofactor which is covalently bound to the protein is known (Johnson and Rajagopalan, 1982; Johnson et al., 1980a,b, 1984; Kramer et al., 1987; Gardlik and Rajagopalan, 1990) and resembles that of many other molybdenum containing oxidoreductases present in various eukaryotic and prokaryotic organisms (Rajagopalan and Johnson, 1992). The enzyme is the product of a single gene (Amaya et al., 1990) and it is present in cells as two interconvertible forms, xanthine dehydrogenase (XD) and xanthine oxidase (XO) (Della Corte and Stirpe, 1968, 1972). The former uses NAD<sup>+</sup> as the acceptor of the reducing equivalents produced during the oxidation of the substrates, whereas the latter transfers the electrons to molecular oxygen (Nishino et al., 1989; Saito and Nishino, 1989). XO is implicated as a possible mediator of tissue damage in many pathological conditions (Parks and Granger, 1983; McCord, 1985; Terada et al., 1991; de Groot and Littauer, 1988) because of its ability to produce superoxide anions as a product of the catalysed reactions (Fridovich, 1970).

Recently we cloned the cDNA coding for the mouse XOR (Terao et al., 1992) and we demonstrated that the expression of the gene is under the control of interferons (IFNs) and IFN-inducing agents both *in vivo* (Terao et al., 1992) and *in vitro* (Falciani et al., 1992). The expression of the XOR transcript was studied in detail in L929 fibroblastic cells (Falciani et al., 1992). This cell line is, in fact, widely used in the assay of the antiviral activity of IFNs and it is thus a biologically relevant experimental model. During the course of studies aimed at clarifying the molecular mechanisms underlying the induction of this enzyme by type I and type II IFNs, we noticed that L929 cells respond to the cytokines with a strong induction of XOR mRNA that does not result in a parallel increase in XD or XO enzymic activities. In L929 cells, the two enzymic activities are undetectable both under basal conditions and after treatment with IFN- $\alpha$  and - $\gamma$  (Falciani et al., 1992).

In the present study we demonstrate that L929 cells contain a pool of XOR inactive protein that is induced after treatment with IFNs. Furthermore, we observe that high concentrations of molybdenum salts are required to activate the enzyme. The activating effect of molybdenum salts is observed in L929, but not in two cell lines where XD and XO activities are expressed under basal conditions and after treatment with IFNs respectively. The data suggest that L929 cells are impaired in the production of the molybdenum cofactor that is essential for the activity of XOR. These results may have important implications in the use of L929 cells as an experimental model to study the pleiotropic activity of IFNs.

Abbreviations used: XOR, xanthine oxidoreductase; XD, xanthine dehydrogenase; XO, xanthine oxidase; IFN, interferon; A.T.C.C., American Type Culture Collection; 1  $\times$  SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.

\* This work is dedicated to the memory of Nella Zambetti-Garattini, a wonderful and understanding mother.

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## MATERIALS AND METHODS

### Cell lines and reagents

L929 is a mouse fibroblastic cell line obtained from the American Type Culture Collection (A.T.C.C.), Rockville, MD, U.S.A. These cells were routinely passaged in RPMI 1640 containing 10% (v/v) fetal-calf serum. The NIH3T3 mouse fibroblasts (A.T.C.C.), the TEnd mouse endothelial cells transformed by the polyoma-virus large T antigen (from Dr. Erwin Wagner, Institute of Molecular Pathology, Vienna, Austria), the COLO26 mouse colon adenocarcinoma (A.T.C.C.), the L1210 mouse lymphocytic leukaemia (A.T.C.C.) and the F9 mouse teratocarcinoma (from Dr. B. Terrana, Sclavo Laboratories, Siena, Italy) were grown in Dulbecco's modified Eagle's medium containing 10% fetal-calf serum. Unless otherwise stated, for the experiments involving treatments with sodium molybdate, cells were allowed to reach confluency before addition of the salt to the growth medium. Under these experimental conditions, sodium molybdate does not have significant effects on the viability of cells. If cells are treated for 24 h, 50 mM is the lowest concentration at which sodium molybdate shows signs of toxicity, producing a cytostatic effect and reducing the viability to 80%. For the experiments involving IFNs, cells were always treated at 50% of their confluency.

Recombinant human IFN- $\alpha$  A/D (*Bg/II*) ( $6.4 \times 10^7$  units/mg) was a gift from Dr. M. Brunda (Hoffmann-La Roche, Nutley, NJ, U.S.A.). Mouse IFN- $\gamma$  ( $10^8$  units/mg) was from Holland Biotechnology (Leiden, The Netherlands). Sodium molybdate, ammonium molybdate and cycloheximide were from Sigma (St. Louis, MO, U.S.A.). All the other reagents were of the highest purity available.

### Measurement of XOR and XO enzymic activities

Cell monolayers from a 25 cm<sup>2</sup> dish were washed twice with 0.9% NaCl, harvested using a 'rubber policeman', and pelleted by centrifugation at 1500 g for 10 min. Cells ( $3 \times 10^6$ ) were resuspended in 60  $\mu$ l of homogenization buffer (0.05 M Tris/HCl, pH 7.8) and disrupted by sonication using a Branson sonifier at its maximum setting, twice, for 5 s at 4 °C. The total homogenate (2–10  $\mu$ l) was used for XOR and XO assays, using [8-<sup>14</sup>C] hypoxanthine (Amersham, Little Chalfont, Bucks., U.K.) as substrate according to the procedure of Reiners et al. (1987). XOR and XO activities were normalized for the content of protein in the sample. Proteins were measured by the method of Bradford (1976), with BSA as a standard. The assay was conducted under conditions of linearity relative to the substrate and to the protein content. A unit of activity is defined as the amount of enzyme capable of transforming 1 nmol of substrate into xanthine and uric acid in 1 min at 37 °C. The detection limit of this enzymic assay is 5 pmol of hypoxanthine transformed into xanthine and uric acid.

### Gel electrophoresis of XOR and Western-blotting analysis

Gel electrophoresis of cell extracts prepared as described in the previous section, transfer of separated proteins to nitrocellulose filters (Stratagene, La Jolla, CA, U.S.A.) and incubation with primary and secondary antibody were carried out according to standard protocols (Maniatis et al., 1989). The primary antibody was kindly given by Dr. T. Nishino (Department of Biochemistry, Yokohama City University, Yokohama, Japan) and it was raised in rabbits against purified rat xanthine dehydrogenase. Preliminary experiments using a 98% pure preparation of mouse XOR isolated as previously described (Carpani et al., 1990)

demonstrated that this antibody specifically recognizes the protein as predicted on the basis of the high level of structural similarity between the rat and the mouse homologues (Amaya et al., 1990; Terao et al., 1992). The secondary antibody was a goat anti-rabbit immunoglobulin fraction linked to horseradish peroxidase (Bio-Rad, Richmond, CA, U.S.A.). Immunoreactive protein bands were revealed by using either radioactive protein G (Amersham) or by a chemiluminescence-based procedure using the ECL detection kit (Amersham) according to the instructions of the manufacturer. Membranes were exposed to Kodak X-Omat X-ray films, and quantification of the XOR protein band was performed with a RAS 3000 video imaging system (Amersham). XOR was revealed in SDS/polyacrylamide gels run under non-reducing conditions by active staining according to the procedure of Waud and Rajagopalan (1976).

### Northern-blotting analysis

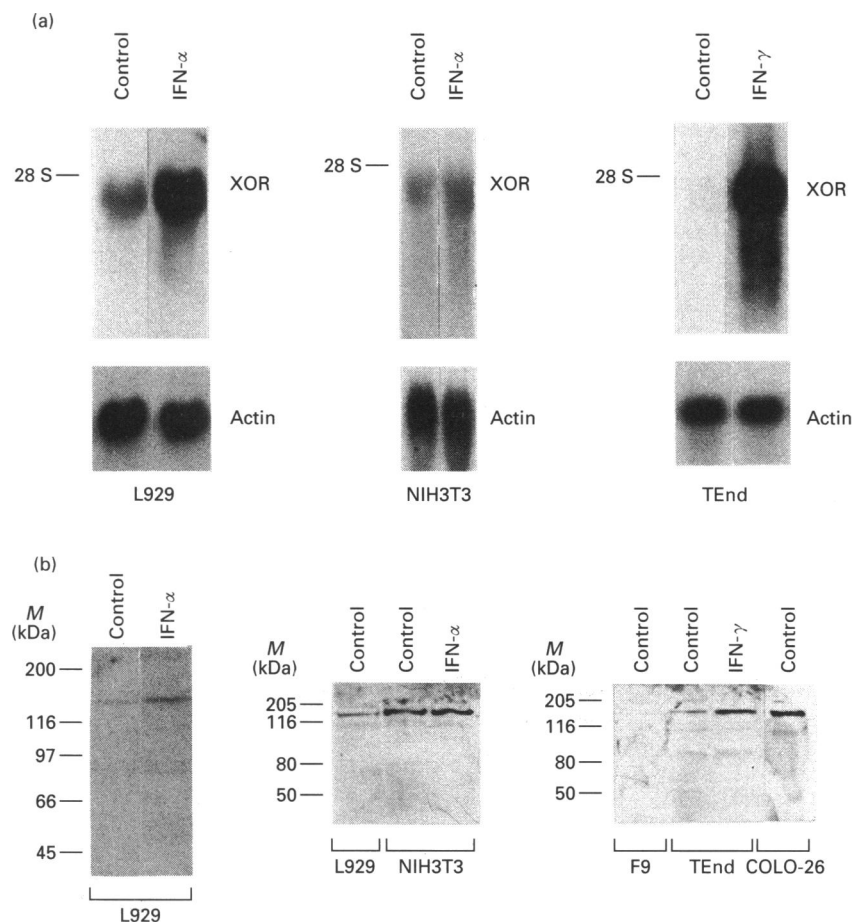
Total RNA was prepared from L929, NIH3T3 and TEnd cells according to a modification of the guanidium isothiocyanate/CsCl method (Rambaldi et al., 1987). RNA (20  $\mu$ g) was then fractionated on a 1.2%-agarose gel with 6% formaldehyde and blotted on to synthetic nylon membranes (GeneScreen Plus; New England Nuclear, Boston, MA, U.S.A.). These membranes were hybridized with XDgt1, a 1.8 kb *Eco*RI fragment of mouse liver XD cDNA (Terao et al., 1992) and they were subsequently hybridized with mouse  $\alpha$ -actin cDNA (Minty et al., 1981). The probes were labelled to a specific radioactivity of  $(1-2) \times 10^9$  c.p.m./ $\mu$ g by using hexanucleotide primers and [<sup>32</sup>P]dCTP (Feinberg and Vogelstein, 1983). Hybridization was performed at 60 °C overnight in a solution containing 1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate (Sigma), 100  $\mu$ g of salmon sperm DNA/ml (Boehringer, Mannheim, Germany) and  $(1-2) \times 10^6$  c.p.m. of labelled probe/ml. The membranes were washed twice with  $2 \times$  SSC/1% SDS ( $1 \times$  SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 30 min at 65 °C and with  $0.1 \times$  SSC for 30 min at room temperature. The membranes were dried and exposed to Kodak X-Omat X-ray films with two intensifying screens (du Pont Cronex; du Pont de Nemours, Bad Homburg, Germany) at -70 °C.

### Determination of total intracellular molybdenum

Cells were seeded in 10 cm<sup>2</sup> Falcon six-well clusters (Beckton Dickinson, Lincoln Park, NJ, U.S.A.) and allowed to reach confluency. After incubation under appropriate experimental conditions and aspiration of the growth medium, cells were scraped off with a rubber policeman in 750  $\mu$ l of 2% HNO<sub>3</sub>. Wells were washed with another 750  $\mu$ l of HNO<sub>3</sub> and the two extracts were combined and centrifuged at 15000 g in a Microfuge (Beckman, Palo Alto, CA, U.S.A.). The determination of <sup>98</sup>Mo was carried out after 100-fold dilution of the extracts in distilled water. All measurements were performed using a Perkin-Elmer Sciex Elan 5000 ICP mass spectrometer (Perkin-Elmer Sciex Instruments, Toronto, Canada). The detection limit for the method is 0.005  $\mu$ g/litre, and recovery of Mo standards varied between 96.7 and 100.2%.

### Complementation of nitrate reductase enzymic activity in extracts of the *nit-1* mutants of *Neurospora crassa* by cell fractions obtained from L929, NIH3T3 and TEnd cells

*nit-1* and wild-type *Neurospora crassa* were obtained from the Fungal Genetics Stock Center (Kansas City, KA, U.S.A.). Strains of *N. crassa* were maintained on 2% (w/v) Bactøagar slopes and grown in liquid culture by following in detail the procedure of



**Figure 1** Effect of IFNs on XOR mRNA and protein in L929, NIH3T3 and TEnd cells

Cells were cultured in the absence or in the presence of 1000 units of human IFN- $\alpha$  A/D or mouse IFN- $\gamma$ /ml for 24 h in the case of RNA analysis and for 48 h in the case of protein analysis. (a) Total RNA was extracted and loaded (20  $\mu$ g/ml) on a 1%-formaldehyde-agarose gel and subjected to Northern-blotting analysis. After transfer, nylon membranes were sequentially hybridized with mouse XOR cDNA and mouse  $\alpha$ -actin cDNA. The position of the 28 S rRNA marker is shown on the left. The XOR mRNA radioactive signals obtained in L929, NIH3T3 and TEnd cells are not directly comparable among themselves because they are representative of separate experiments. (b) Cells were collected by trypsin treatment, homogenized and subjected to Western-blotting analysis using an XOR-specific polyclonal antibody. The immunoreactive band was revealed by autoradiography after incubation with  $^{125}$ I-labelled protein G (left) or by the use of a chemiluminescent substrate after incubation with the secondary antibody linked to horseradish peroxidase (middle and right). Western-blotting analysis was conducted with the same amount of protein (335  $\mu$ g) extracted from mouse L929 fibroblastic cells, NIH3T3 cells, TEnd endothelial cells and COLO-26 colon carcinoma cells, incubated for 24 h in the absence (Control) or in the presence of the indicated cytokines. F9 teratocarcinoma represent a negative control for the expression of the XOR protein. Abbreviation: *M*, molecular mass.

Nason et al. (1970). The *nit-1* mutant cells were transferred to  $\text{NO}_3^-$  containing induction medium before being harvested. Mycelia were centrifuged and stored at  $-80^\circ\text{C}$  until use. Extracts of mycelia were obtained after homogenization in 0.5 mM EDTA/1 mM dithiothreitol/0.1 mM phenylmethanesulphonyl fluoride/100 mM potassium phosphate, pH 7.2, with a Teflon/glass Potter-Elvehjem homogenizer. Homogenates were centrifuged at 15000 *g* for 10 min at  $4^\circ\text{C}$  and the supernatant (30  $\mu$ l/each assay) was immediately used for the complementation assay. L929, NIH3T3 and TEnd cells obtained from three 75  $\text{cm}^2$  culture flasks (approx.  $3 \times 10^7$  cells) were homogenized using a Branson sonifier in 10 mM potassium phosphate, pH 7.4. The homogenate (30  $\mu$ l) was used for the complementation assay. The complementation reaction was carried out overnight at  $4^\circ\text{C}$  in the absence or in the presence of 1 mM sodium molybdate. Under the experimental conditions used, increasing concentrations of cell extracts gave a linear response in terms of activation of the *N. crassa* nitrate reductase. The measurement of nitrate reductase activity was according to Amy and Rajagopalan (1979) and results are expressed as the change in absorbance at

540 nm/min, which is proportional to the amount of nitrite formed during the reaction. The results are normalized for the protein content in the cell extracts.

## RESULTS

The expression of the XOR gene after treatment with type I or type II IFNs was studied in L929, NIH3T3 and TEnd cells. These cell lines were chosen because they have different patterns of expression of the XOR gene in basal conditions and after treatment with IFNs. As shown in Figure 1(a) (left), the L929 fibroblastic cell line presents low levels of expression of the XOR mRNA under basal conditions and it responds to IFN- $\alpha$  A/D with an approx. 5-fold induction of the transcript. The mRNA induction is accompanied by a similar increase in XOR protein as determined by Western-blot analysis using a rabbit polyclonal antibody raised against rat XOR, which cross-reacts with the mouse homologue (Figure 1b, left). However, as shown in Table 1, the XOR enzyme, determined as the sum of XD and XO

**Table 1** Effect of IFN- $\alpha$  (A/D) and IFN- $\gamma$  on the levels of XOR enzymic activity

Cells were cultured in the absence or in the presence of 1000 units of human IFN- $\alpha$  A/D or mouse IFN- $\gamma$ /ml for 48 h. Cells were collected by scraping with a rubber policeman and homogenized as described in the Materials and methods section; 10  $\mu$ l of the homogenate was incubated with [ $^{14}$ C]hypoxanthine for 10 min in the presence of NAD $^{+}$ . XOR total activity is determined as the sum of XD and XO enzymic activities after quantification of the amount of [ $^{14}$ C]xanthine and uric acid formed; 0.01 unit/mg of protein is the limit of detection of the enzymic assay. Abbreviation: N.D., not done. Each experimental value is the mean  $\pm$  S.D. from three separate culture dishes.

Treatment	XOR activity (units/mg of protein)			
	L929	NIH3T3	TEnd	COLO26
None	< 0.01	18.83 $\pm$ 1.94	0.12 $\pm$ 0.01	11.71 $\pm$ 0.23
IFN- $\alpha$	< 0.01	18.24 $\pm$ 2.31	0.11 $\pm$ 0.02	N.D.
IFN- $\gamma$	< 0.01	17.63 $\pm$ 1.32	1.98 $\pm$ 0.10	N.D.

**Table 2** Effect of sodium molybdate on XOR enzymic activity in L929, NIH3T3 and TEnd cells after incubation in the absence and in the presence of IFNs

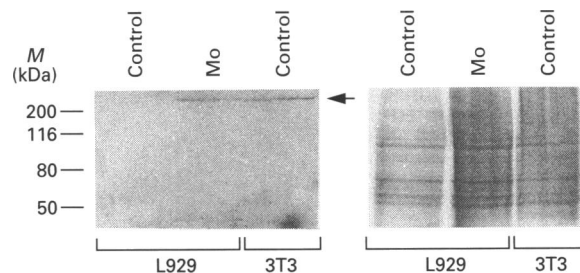
L929, NIH3T3 and TEnd cells were incubated in the absence or in the presence of 1000 units of human IFN- $\alpha$  A/D or mouse IFN- $\gamma$ /ml for 30 h and subsequently the cells were treated with medium alone (none), medium containing 10 mM sodium molybdate (Mo), medium containing 1000 units of human IFN- $\alpha$  A/D/ml (IFN- $\alpha$ ), medium containing 1000 units of mouse IFN- $\gamma$ /ml (IFN- $\gamma$ ) or medium containing the combination of the two compounds (either IFN- $\alpha$  + Mo or IFN- $\gamma$  + Mo) for another 18 h. Cells were harvested by scraping with a rubber policeman, homogenized, and an appropriate aliquot of the homogenate was used for the assay of total XOR enzymic activity determined as the sum of XD and XO activities; 0.01 unit/mg of protein is the limit of detection of the enzymic assay. Abbreviation: N.D., not done. Each experimental value is the mean  $\pm$  S.D. from three separate culture dishes.

Treatment	XOR enzymic activity (units/mg of protein)		
	L929	NIH3T3	TEnd
None	< 0.01	11.11 $\pm$ 1.34	0.10 $\pm$ 0.01
Mo	0.39 $\pm$ 0.03	11.42 $\pm$ 1.51	0.13 $\pm$ 0.03
IFN- $\alpha$	< 0.01	13.92 $\pm$ 3.95	0.12 $\pm$ 0.02
IFN- $\alpha$ + Mo	0.94 $\pm$ 0.06	11.25 $\pm$ 0.54	N.D.
IFN- $\gamma$	< 0.01	12.40 $\pm$ 0.82	0.74 $\pm$ 0.08
IFN- $\gamma$ + Mo	N.D.	N.D.	0.69 $\pm$ 0.06

activities, is undetectable both under basal conditions and after treatment with IFN- $\alpha$  A/D or IFN- $\gamma$ .

The inability to measure XOR enzymic activity is not due to lack of sensitivity of the assay used. In fact, L929 expresses approximately the same amount of XOR mRNA and immunoreactive protein as TEnd, a cell line that expresses measurable levels of XOR activity (see below). Moreover, XOR enzymic activity in L929 cells is not measurable even if a quantity of protein more than ten times higher than that of TEnd cells is used for the assay (results not shown).

As shown in Figure 1(a) (middle), Figure 1(b) (middle) and in Table 1, NIH3T3 fibroblasts express significant amounts of XOR message, immunoreactive protein and enzymic activity under basal conditions. Densitometric analysis of the signal corresponding to the XOR immunoreactive band demonstrates that NIH3T3 has approx. 10-fold higher levels of the protein

**Figure 2** Induction of a single protein with XO activity by sodium molybdate

L929 and NIH3T3 fibroblastic cells were grown for 24 h in the absence (control) or in the presence (Mo) of 10 mM sodium molybdate. Cells were harvested by scraping them off the wells, homogenized, and the resulting extracts containing 300  $\mu$ g of protein were subjected to 7.5% SDS/PAGE under non-reducing conditions. Gels were stained with NitroBlue Tetrazolium in the presence of hypoxanthine for the active staining of xanthine (left panel) and with Coomassie Blue for the detection of proteins. The arrow indicates the position of the band with XO enzymic activity. The positions of molecular-mass ( $M$ ) standards are indicated on the left.

relative to L929 cells grown in basal medium. In NIH3T3 cells, although IFN- $\alpha$  A/D increases the expression of the XOR mRNA by 1.5-fold, the respective protein is not significantly induced. Similar results were obtained after treatment of L929 cells with IFN- $\gamma$  (data not presented).

As shown on Figure 1(a) (right), TEnd expresses high levels of XOR mRNA after treatment for 24 h with IFN- $\gamma$  (but not with IFN- $\alpha$  A/D; results not shown). This cell line expresses XOR mRNA also under basal conditions, since a specific signal for the transcript is observed in the control lane when the Northern blot is exposed for a longer period of time (results not shown). Figure 1(b) (right) demonstrates that the induction of the expression of the XOR gene is accompanied by an augmentation of the respective protein, even though the response (approx. 8-fold increase) is lower than expected on the basis of the up-regulation of the mRNA. In TEnd cells, the enzymic activity (Table 1) is low, but readily detectable, and it is stimulated by approx. 20-fold after treatment with IFN- $\gamma$  for 24 h. F9 teratocarcinoma and COLO26 adenocarcinoma cell extracts are shown in Figure 1(b) (right) as negative and positive controls respectively, to demonstrate the specificity of the XOR band detected in Western-blotting analysis. In fact, F9 cells do not show detectable levels of XOR enzymic activity before and after treatment with IFN- $\alpha$  A/D or IFN- $\gamma$  (results not shown), while COLO26 expresses amounts of the enzyme that are similar to those observed in NIH3T3 (Table 1).

Since XD and XO are known to depend on the presence of a molybdopteridine cofactor for their activity (Rajagopalan and Johnson, 1992), the effect of molybdenum on L929 XOR was studied. As shown in Table 2, addition of sodium molybdate to the culture medium at a concentration of 10 mM is capable of inducing XOR activity (measured as the sum of XD and XO) both under basal conditions and after treatment for 24 h with IFN- $\alpha$  A/D. Similar results are obtained when sodium molybdate is replaced by ammonium molybdate (results not shown). The action of molybdenum is not limited to the L929 clone routinely passaged in our laboratory, since it is consistent with another L929 clone obtained from a different laboratory (Dr. Santo Landolfo, Istituto di Microbiologia, University of Turin, Turin, Italy), and a virally infected L929 clone (Radaelli et al., 1984) (results not shown). However, it is specific for mouse L929 derivative lines, since the salt is not affecting XOR activity in

**Table 3** Effect of allopurinol on the induction of XOR enzymic activity by sodium molybdate in L929 cells

L929 fibroblastic cells were treated for 24 h with medium alone (None), medium containing the indicated concentrations of allopurinol (Allo), medium containing 10 mM sodium molybdate (Mo) or with medium containing both compounds (Allo+Mo) as indicated. Cells were harvested by scraping with a rubber policeman, homogenized, and an appropriate aliquot of the homogenate was used for the assay of both total XOR (XD+XO) and XO enzymic activities; 0.01 unit/mg of protein is the limit of detection of the enzymic assay. Each experimental value is the mean  $\pm$  S.D. from three separate culture dishes.

Treatment	Enzymic activity (units/mg of protein)	
	XO + XD	XO
None	< 0.01	< 0.01
Allo (0.1 mM)	< 0.01	< 0.01
Allo (1 mM)	< 0.01	< 0.01
Mo	0.35 $\pm$ 0.03	0.12 $\pm$ 0.02
Allo (0.1 mM) + Mo	0.07 $\pm$ 0.01	0.02 $\pm$ 0.01
Allo (1 mM) + Mo	< 0.01	< 0.01

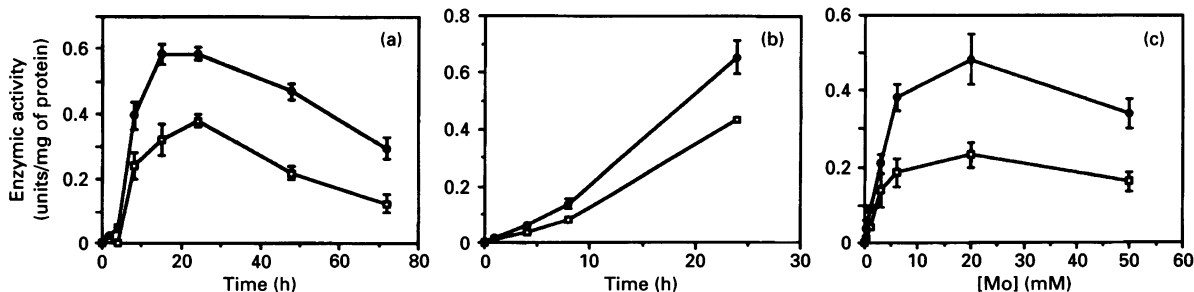
NIH3T3 or TEnd cells under any experimental condition (Table 2). Other mouse and human cell lines, such as F9 teratocarcinoma, L1210 lymphocytic leukaemia, COLO26 adenocarcinoma, HEPG2 hepatoma and HUVEC umbilical-vein endothelial cells, are not responsive to sodium molybdate treatment in terms of XOR activity (results not shown). These cell types do not present XOR enzymic activity in basal conditions, except for mouse COLO26 adenocarcinoma.

Figure 2 demonstrates that the activation of XOR enzymic activity by sodium molybdate in L929 cells is accompanied by the induction of a specific protein band with XO activity, migrating with an apparent molecular mass of 300 kDa on SDS/PAGE under non-reducing conditions. In these experimental conditions, purified XOR migrates as an enzymically active dimer (Carpani et al., 1990). A similar band is observed in extracts of NIH3T3 cells grown in the absence of molybdenum. Approximately the same amount of protein was loaded in all the lanes of the gel, as demonstrated by the Coomassie Blue staining of a similar gel run in parallel (right-hand side of the Figure). As demonstrated by Table 3, the induced XOR activity is inhibited

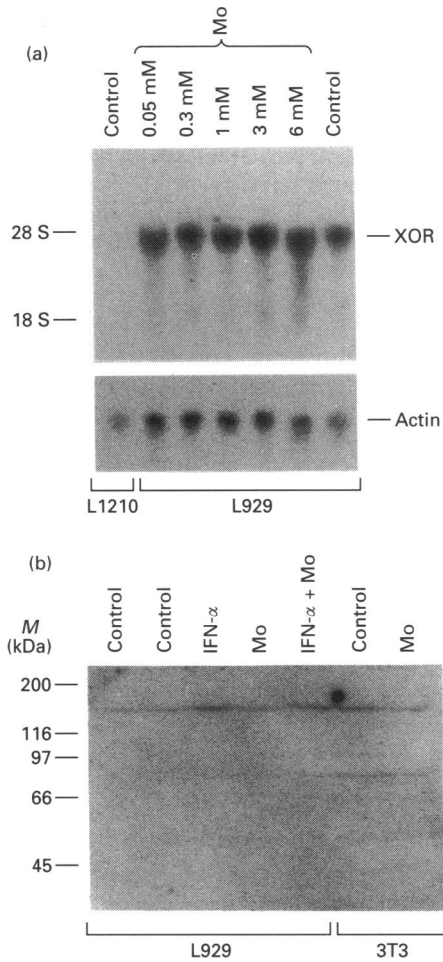
by addition to the culture medium of allopurinol (0.1 and 1 mM), a specific irreversible inhibitor of XD and XO (the level of inhibition is similar for both total XOR and XO activities). At these two concentrations, allopurinol is not toxic as judged by the morphology, the growth and the viability of L929 cells. Taken together, these data prove that a *bona fide* XOR enzyme is induced by molybdenum salts.

The activation of XOR enzyme is observed when molybdenum is added to the cell cultures, but not to the cell homogenates. In fact, if L929 cells are disrupted by sonication and incubated in the presence of sodium molybdate (50  $\mu$ M) for 2 h at room temperature or for 12 h at 4  $^{\circ}$ C, XOR is not detectable. Under these experimental conditions the enzyme is stable, since XOR activity does not change in NIH3T3 cell extracts. In fact if NIH3T3 extracts are incubated for 2 h at room temperature, the XOR activity observed is 10.7  $\pm$  0.8 in the absence and 10.5  $\pm$  0.6 units/mg of protein in the presence of sodium molybdate, whereas after 12 h at 4  $^{\circ}$ C, the values are 10.0  $\pm$  0.3 and 10.5  $\pm$  0.2 respectively. Similar results for L929 and NIH3T3 are obtained when sodium molybdate is increased to 5 mM.

The time course and the dose response for the induction of XOR activity in L929 cells are presented in Figure 3. In these experiments, total XOR (XD+XO) and XO enzymic activities were measured separately. As shown in Figure 3(a), sodium molybdate at a concentration of 10 mM induces both XO and XOR activity with similar kinetics, and the ratio XO/XOR (approx. 50%) is not changed at any time point. The kinetics of induction of XOR and XO enzymic activities are relatively slow. Maximal increase is observed at 16 h, and the two activities remain high until 24 h. At 48 h a decrease in the levels of XOR and XO is observed, probably as a consequence of the mild toxicity produced by prolonged treatment with high concentrations of sodium molybdate. Figure 3(b) demonstrates that the continuous presence of the molybdenum salt in the medium is required to obtain maximal activation of XOR and XO. If cells are incubated in the presence of 10 mM sodium molybdate for 1, 4 and 8 h before change to fresh medium without molybdenum salt and further incubation up to 24 h, submaximal activation of the two enzymes is observed (compare the values obtained at these time points relative to that obtained after treatment of cells for 24 h in the presence of sodium molybdate). Figure 3(c) shows that maximal induction of XOR and XO is observed after incubation of cells for 24 h in the presence of 20 mM sodium molybdate, even though detectable amounts of the two enzymic

**Figure 3** Effect of the exposure time and concentration of sodium molybdate on the induction of XOR enzymic activity in L929 cells

(a) Cells were treated with 10 mM sodium molybdate for the indicated amount of time. Cells were scraped from culture dishes, homogenized, and an aliquot of the homogenate was used for the assay of total XOR (●) or XO (□) enzymic activity. (b) Cells were treated with 10 mM sodium molybdate for the indicated amount of time, washed and incubated with fresh medium without the molybdenum salt for up to 24 h. Cells were harvested and used for the determination of total XOR (●) and XO (□) enzymic activities as in (a). (c) Cells were treated with the indicated amounts of sodium molybdate for 24 h. Measurement of total XOR (●) and XO (□) enzymic activities were performed as described in (a). Each experimental value is the mean  $\pm$  S.D. for three separate culture dishes.



**Figure 4** Effects of sodium molybdate on the steady-state levels of XOR mRNA and protein

(a) L929 cells were treated for 24 h with the indicated concentrations of sodium molybdate. Total RNA was extracted and loaded (20  $\mu\text{g}/\text{ml}$ ) on a 1% formaldehyde agarose gel and subjected to Northern-blotting analysis. After transfer, nylon membranes were sequentially hybridized with mouse XOR cDNA and mouse  $\alpha$ -actin cDNA. The position of the 28 S ribosomal RNA marker is shown on the left. RNA extracted from the mouse L1210 cell line was used as a negative control. (b) L929 cells were treated for 24 h with 1000 units of IFN- $\alpha$  A/D/ml (IFN- $\alpha$ ), 2 mM sodium molybdate (Mo) or a combination of the two compounds. 3T3 cells were incubated in the absence (Control) or in the presence of 2 mM sodium molybdate (Mo). Cells were homogenized, and an aliquot of the homogenates containing 300  $\mu\text{g}$  of protein was subjected to Western-blotting analysis using polyclonal antibodies specific for XOR. The immunoreactive protein band was revealed by autoradiography after incubation with  $^{125}\text{I}$ -labelled protein G. Abbreviation: M, molecular mass.

activities are already evident after incubation with 500  $\mu\text{M}$ . No further activation of the two enzymes is observed if the concentration of the molybdenum salt is raised up to 50 mM.

As shown in Figure 4(a), Northern-blotting experiments, performed on total RNA extracted from L929 cells incubated for 24 h in the presence of increasing concentrations of sodium molybdate, demonstrate that the steady-state levels of XOR mRNA are not influenced by molybdenum. Thus the metal does not induce XOR enzymic activity through an increase in the expression of the corresponding gene. Furthermore, Figure 4(b) rules out the possibility that the induction of XOR enzyme by molybdenum is the consequence of increased translation from the respective transcript. In fact, the levels of the 150 kDa XOR

**Table 4** Effect of cycloheximide on the levels of XOR enzymic activity in L929 cells

L929 cells were cultured for 30 min in medium alone or in medium containing 20  $\mu\text{g}$  of cycloheximide (CHX)/ml, followed by 6 h in the absence (None) or presence of 10 mM sodium molybdate (Mo). CHX + Mo\* indicates cells incubated simultaneously for 6 h with the combination of cycloheximide and Mo. Cells were homogenized, and an aliquot of the homogenates was used for the determination of total XOR enzymic activity; 0.01 unit/mg of protein is the limit of detection of the enzymic assay. Each experimental value is the mean  $\pm$  S.D. from three separate culture dishes.

Treatment	XOR enzymic activity (units/mg of protein)
None	< 0.01
Mo	0.52 $\pm$ 0.03
CHX	< 0.01
CHX + Mo*	0.40 $\pm$ 0.09
CHX + Mo	0.80 $\pm$ 0.10

**Table 5** Effect of the exposure time of sodium molybdate in the growth medium on the intracellular level of molybdenum in L929, NIH3T3 and TEnd cells

Cells were exposed to 5 mM sodium molybdate for the indicated amount of time. The intracellular concentration of molybdenum (Mo) was determined. Each experimental value is the mean  $\pm$  S.D. from three separate culture dishes.

Time	[Mo] ( $\mu\text{g}/10^6$ cells)		
	L929	NIH3T3	TEnd
0 min	0.37 $\pm$ 0.02	0.82 $\pm$ 0.04	0.43 $\pm$ 0.04
10 min	1.07 $\pm$ 0.12	1.95 $\pm$ 0.02	1.23 $\pm$ 0.03
30 min	1.82 $\pm$ 0.13	2.67 $\pm$ 0.18	1.89 $\pm$ 0.14
2 h	1.36 $\pm$ 0.08	2.99 $\pm$ 0.26	1.80 $\pm$ 0.06
24 h	1.27 $\pm$ 0.11	3.45 $\pm$ 0.29	1.91 $\pm$ 0.13
48 h	1.54 $\pm$ 0.03	1.76 $\pm$ 0.09	2.10 $\pm$ 0.02

immunoreactive protein are not changed after incubation of L929 cells with 2 mM sodium molybdate, a concentration that potentially activates XOR enzymic activity. Finally, as shown in Table 4, the induction of XOR enzymic activity is not blocked by incubation of the L929 cells with cycloheximide at concentrations known to inhibit protein synthesis. At this concentration, cycloheximide has a slight inducing effect on XOR enzymic activity if cells are preincubated with the protein-synthesis inhibitor for 30 min before addition of sodium molybdate. Thus *de novo* protein synthesis is not required for the inducing effect triggered by sodium molybdate.

To study further the defect of L929 cells leading to the production of an inactive form of XOR apoprotein, the time course and the concentration curve for the intracellular molybdenum was compared with those observed in NIH3T3 and TEnd cells. As shown in Table 5, after incubation with 5 mM sodium molybdate, the accumulation of the metal is similar in L929 relative to TEnd and NIH3T3 cells. Maximal levels of molybdenum are evident after 30 min in the three cell lines. However, NIH3T3 accumulate an approx. 2-fold higher amount of molybdenum relative to the other two cell lines at 24 h. Moreover, the intracellular levels of molybdenum in L929 and TEnd are similar and approx. 50% lower than those observed in NIH3T3, in the absence of sodium molybdate in the growth medium (0 min). Table 6 demonstrates that the accumulation of

**Table 6** Effect of the concentration of sodium molybdate in the growth medium on the intracellular level of molybdenum in L929, NIH3T3 and TEnd cells

Cells were exposed to the indicated concentrations of sodium molybdate for 24 h. The intracellular concentration of molybdenum (Mo) was determined. Each experimental value is the mean  $\pm$  S.D. from three separate culture dishes.

Concn. (mM)	[Mo] ( $\mu\text{g}/10^6$ cells)		
	L929	NIH3T3	TEnd
0	0.29 $\pm$ 0.05	1.12 $\pm$ 0.31	0.37 $\pm$ 0.03
0.1	0.30 $\pm$ 0.02	1.09 $\pm$ 0.34	0.52 $\pm$ 0.05
1.0	2.97 $\pm$ 1.84	6.72 $\pm$ 1.23	4.62 $\pm$ 0.27
10	5.64 $\pm$ 1.36	15.63 $\pm$ 6.85	9.25 $\pm$ 2.63
50	16.68 $\pm$ 5.54	49.13 $\pm$ 4.15	12.01 $\pm$ 1.37

**Table 7** Effect of sodium molybdate on the capacity of L929- and NIH3T3-cell extracts to restore nitrate reductase enzymic activity of the *nit-1* mutant of *Neurospora crassa*

L929 and NIH3T3 cells were exposed to medium alone (L929; NIH3T3) or medium containing 5 mM sodium molybdate [L929 (Mo); NIH3T3 (Mo)] for 24 h. Cells were harvested, homogenized, and an aliquot of the homogenate containing the same amount of protein (180  $\mu\text{g}$ ) was incubated overnight at 4  $^{\circ}\text{C}$  with extracts of mycelia from *nit-1* *Neurospora crassa* in the absence (– Mo) or in the presence (+ Mo) of 1 mM sodium molybdate. Appropriate blanks of *nit-1* extracts (*nit-1*) were incubated in parallel with the samples in the presence or in the absence of Mo (– Mo; + Mo). Nitrate reductase activity was measured in reconstituted extracts, and the results are normalized for the amount of protein present in the L929- and NIH3T3-cell homogenates. Values are expressed as  $\Delta A_{540}/\text{min}$  per mg of protein in the eukaryotic-cell extract. < 1.0, below the limit of detection of the assay. Each experimental value is the mean  $\pm$  S.D. for three assays.

Extract	Nitrate reductase activity	
	– Mo	+ Mo
<i>nit-1</i>	< 1.0	< 1.0
L929 + <i>nit-1</i>	< 1.0	42.8 $\pm$ 2.5
L929 (Mo) + <i>nit-1</i>	26.6 $\pm$ 2.1	57.8 $\pm$ 3.0
NIH3T3 + <i>nit-1</i>	24.6 $\pm$ 1.7	47.5 $\pm$ 1.7
NIH3T3 (Mo) + <i>nit-1</i>	10.4 $\pm$ 0.6	28.8 $\pm$ 2.4

molybdenum is similar for all the three lines, even though NIH3T3 retains 2–3-fold more molybdenum than L929 or TEnd cells. The results are consistent when expressed on the basis of the protein content in the various cells. Moreover, the average volume of L929 relative to NIH3T3 and TEnd cells is very similar, as assessed by direct measurement in a Coulter counter (results not shown).

The relative amount of intracellular molybdenum cofactor which is essential for the activity of XOR was next evaluated. For this purpose, extracts of L929 and NIH3T3 cells grown in the presence and in the absence of 5 mM sodium molybdate were tested for their ability to complement the nitrate reductase defect observed in extracts of the *nit-1* mutant of *N. crassa*. This complementation test is widely used to assess the presence of molybdenum cofactor in an indirect way (Johnson, 1980). In the absence of molybdenum exogenously added to the reconstitution mixture, the assay allows the determination of the levels of the preformed molybdenum cofactor present in the mouse cell lines (Ketchum and Swamin, 1973). In this case, as shown in Table 7, extracts obtained from NIH3T3 cells grown either in the absence

or in the presence of sodium molybdate rescue *N. crassa* nitrate reductase activity. By contrast, L929 cells grown in the presence of molybdenum rescue nitrate reductase activity, whereas those grown in the absence of the metal do not. If the complementation assay is carried out in the presence of molybdenum (1 mM) exogenously added to the reconstitution mixture, an enzymic activity (the converting enzyme) capable of metabolizing precursors of the molybdenum cofactor that accumulate in the cells of the *N. crassa nit-1* mutants is preferentially measured (Johnson et al., 1989; Rajagopalan and Johnson, 1992). Under these experimental conditions it is evident from Table 7 that both L929 cells and NIH3T3, grown either in the absence or in the presence of sodium molybdate, can rescue *nit-1* nitrate reductase activity. The reconstitution experiments involving complementation of the *N. crassa* extracts for nitrate reductase activity were performed using crude extracts of the micelia. Qualitatively similar results were obtained if the fungal extract was passed through a Sephadex G-25 column in order to obtain a nitrate reductase preparation free of the low-molecular-mass pteridine precursors (results not shown).

## DISCUSSION

The data presented here demonstrate that mouse L929 fibroblasts have a defect in the assembly of active XOR both under basal conditions and after treatment with type I or type II IFNs. The enzymic activity is rescued by the addition of millimolar concentrations of sodium molybdate to the growth medium. The activation of XOR by molybdenum salts is specific for mouse L929 fibroblasts, since it is not observed in a panel of other mouse and human cell lines. Our experimental data suggest that L929 cells represent a mutant for the expression of XOR. The deficit, however, cannot be explained by a structural alteration of the XOR protein, but rather by impaired synthesis of the molybdenum cofactor that is essential for the activity of the enzyme. As demonstrated by the use of a heterologous complementation test, L929 cells have undetectable levels of molybdenum cofactor if grown in the absence of sodium molybdate. If the cells are grown in the presence of the molybdenum salt, the cofactor can be measured using the same complementation test. NIH3T3 cells which are fully competent for the expression of XOR enzyme complement the *nit-1* nitrate reductase deficit, regardless of whether they are grown in the absence or in the presence of sodium molybdate.

Molybdenum-cofactor biosynthesis and metabolism have been studied in eukaryotes (Johnson et al., 1989) and more extensively in prokaryotes (Stewart, 1988). In eukaryotes, most of the information on the biosynthesis of the molybdenum cofactor have been gathered from studies on fibroblasts obtained from human patients suffering from a rare genetic disease known as 'combined deficiency of sulphite oxidase and xanthine oxidase' (Johnson and Wadman, 1989). The symptoms of this disease are predominantly the consequence of the sulphite oxidase defect, which leads to a severe impairment of the central nervous system (Aukett et al., 1988; Endres et al., 1988). At present, two complementation groups, A and B (Johnson et al., 1989), have been characterized. Group-A patients are unable to accumulate molybdopterin precursors as a consequence of unknown metabolic defects in this biosynthetic pathway, whereas group-B patients are unable to metabolize molybdopterin precursors to molybdenum cofactor because of a lack of converting enzyme (Johnson et al., 1989). The converting enzyme is a protein catalysing the transformation of the precursor Z (Rajagopalan and Johnson, 1992) to a pteridin compound which subsequently interacts with molybdenum and produces the molybdenum

cofactor. This is exactly the same enzymic activity that is lacking in the *nit-1* mutants of the *N. crassa* (Rajagopalan and Johnson, 1992) that we used in the present study. In prokaryotes, the metabolism of molybdenum cofactor has been studied both in terms of its genetics and biochemistry on several pleiotropic mutants of *Escherichia coli* selected as chlorate-resistant strains (Stewart, 1988). In bacterial cells, several genes are directly involved in the biosynthetic pathway leading to the formation of active molybdenum cofactor. The *chlA* genes 4 and 5 encode the two subunits that constitute the active form of the converting enzyme, whereas the *chlN* gene is involved in the activation of the small subunit of the complex (Reiss et al., 1987; Pitterle and Rajagopalan, 1989; Pitterle et al., 1990; Zurick et al., 1991). The *chlD* and *chlG* gene products are involved in the transport across the membrane and possibly in the insertion of the molybdenum ions in the pteridine precursor respectively (Stewart, 1988; Glaser and DeMoss, 1971). In fact, *chlD* mutants are defective in one or more factors involved in the transport of molybdenum inside the cell (Stewart, 1988). By contrast, the *chlG* mutant seems to express an altered form of a putative intracellular carrier protein necessary to donate molybdenum to compound Z, the immediate precursor of molybdenum cofactor (Glaser and DeMoss, 1971).

L929 cells are certainly not defective in the expression of the converting enzyme. In fact, extracts of this cell line, grown both in the absence and in the presence of sodium molybdate, can rescue *N. crassa* nitrate reductase if the complementation test is performed in the presence of exogenously added molybdenum(VI) ions. These complementation conditions are known to allow the indirect measurement of the converting enzyme (Johnson et al., 1989; Johnson and Rajagopalan, 1992).

Insofar as the XOR enzymic activity is restored by treatment in high concentrations of sodium molybdate, L929 cells resemble the *chlD* and *chlG* mutants of *E. coli* as well as the *nifQ* mutants of *Klebsiella pneumoniae* (Imperial et al., 1984). The *chlD* mutant of *E. coli* and the *nifQ* mutant of *K. pneumoniae* are defective in the transport of molybdenum inside the cell. Unequivocal demonstration of a lesion in the transport of molybdenum across the cell membrane would require experiments of intracellular accumulation of the metal using fast kinetics and concentrations of the metal similar to those observed in the growth medium without supplementation of sodium molybdate. However, two pieces of evidence suggest that the deficit in the expression of XOR activity in L929 cells is not the result of a defective intracellular accumulation of molybdenum. *chlD* mutant cells contain approx. 10–20 % of the levels of molybdenum present in wild-type *E. coli*, whereas in the absence of sodium molybdate in the growth medium, L929 fibroblasts have intracellular levels of the metal that are lower than those observed in NIH3T3, but similar to those observed in TEnd endothelial cells, which are competent for the expression of XOR. Moreover, at concentration of Mo between 0.1 and 50 mM, the kinetics and the concentration profile for the intracellular accumulation of molybdenum seem to be similar in the three cell lines. Although the absolute amounts of intracellular molybdenum in NIH3T3 are higher than those in L929 and TEnd, clearly this fact alone does not explain the observed differences in the expression of XOR.

At present we do not have any direct evidence to explain the mechanism underlying the defect observed in L929 cells, even though it is quite possible that a factor or an enzyme involved in one of the several steps leading to the synthesis of molybdenum cofactor is altered in its structure and it requires high concentrations of molybdenum to work. This would account for the facts that the molybdenum cofactor does not accumulate in the intracellular compartment, even though the molybdenum trans-

port across the membrane is normal. It would be tempting to speculate that the deficit observed in L929 cells is analogous to that observed in *ChlG* mutants of *E. coli*.

Since the molecular defect observed in L929 probably results in the synthesis of the molybdenum cofactor, it is anticipated that this cell line must be also deficient in the expression of sulphite oxidase and aldehyde oxidase as well. We are currently trying to address this issue, even though the low sensitivity of the spectrophotometric assays used for the determination of these two enzymic activities and the lack of cDNA clones coding for the two proteins in mammals make these studies difficult to perform in L929 cells.

It would be of considerable interest to know whether the defect in the expression of XOR observed in L929 is a genetic trait acquired by the process of immortalization and stabilization *in vitro* of the cell line or whether the defect was already present in the original mouse strain. In fact, this cell line has been derived more than 50 years ago from normal subcutaneous areolar and adipose tissue of a 100-day-old male C3H/An mouse. Mice from the syngeneic strain C3H/HeN do not present a deficit of XO in the liver (results not shown). By contrast, it is known that this mouse strain contains approximately one-tenth of the amount of aldehyde oxidase present in C57BL/6 mice (Holmes et al., 1981; Huff and Chaykin, 1967). Unfortunately, this evidence does not allow us to draw any conclusion, especially in view of the fact that the situation in the whole animal is certainly different from that observed in cell culture. Moreover, it is to be remembered that laboratory animals are fed with semi-synthetic diets which contain molybdenum ions, and the daily requirement for this metal is extremely low (Rajagopalan, 1988).

In conclusion, in spite of the fact that the mechanism underlying the defect of XOR expression in L929 is still not completely understood, the findings presented here have a potential and general interest at least in two areas of investigation.

First of all, the correction of the XOR defect in L929 cells by molybdenum salts might have some clinical relevance for the aetiology and the treatment of the combined deficiency of molybdoenzyme activities. So far, all the patients suffering from this disease appear to be deficient in molydopterin rather than molybdenum itself (Rajagopalan, 1988). Although administration of molybdenum proved ineffective to correct the deficit in one reported case, this need not be true in all instances. To the best of our knowledge, the effect of molybdenum salts on the deficit of XOR and sulphite oxidase activity observed in primary fibroblast obtained from these patients has never been tested systematically. It is thus possible that a subgroup of group-A-type fibroblasts might be responsive to molybdenum challenge if the underlying defect is similar to that observed in mouse L929 cells. For these reasons, it would be interesting to test the effect of high concentrations of the metal on XOR or sulphite oxidase activities in group-A fibroblasts. It would be also worthwhile performing cell-fusion experiments between human group-A and group-B mutants and mouse L929 cells to investigate the relative complementation pattern.

Secondly, our findings are of concern to those investigators involved in the study of the biological effects produced by IFNs. In fact, although it is yet to be established that XOR protein plays any role in the immunomodulatory, antiviral or antiproliferative activities triggered by these cytokines, it is clear that the relative gene is regulated by both IFN- $\alpha$  and - $\gamma$  without *de novo* synthesis of intermediary transcription factors (Terao et al., 1992; Falciani et al., 1992). As such the XOR gene should be considered as an IFN-responsive one. Since L929 cells are largely used for the study of both the antiviral and the antiproliferative activity of IFNs, it should be emphasized that this cell line is



defective in the expression of at least one of the possible genes involved in the pleiotropic response to these cytokines.

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